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LAVAGE OF LEUKOTRIENE B<sub>4</sub> INDUCES LUNG GENERATION OF TUMOR  
NECROSIS FACTOR-A AND NEUTROPHIL DIAPEDESIS

BY

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with peak concentrations of 580 pg/ml after 1.5 hours and 120 pg/ml after 3 hours. These values were higher than following lavage of 0.1 ml saline, 0.7 pg/ml and 4.3 pg/ml respectively (both  $p < 0.05$ ). There was a delay before PMN accumulated in BAL fluid ( $\times 10^4$ ). After 30 min the numbers were 2.2 PMN/ml while at 4 hours there was a rise to 40 PMN/ml and at 5 hours 60 PMN/ml, higher than following saline lavage (all  $p < 0.05$ ). Pretreatment of rats by lavage into airways of actinomycin D, 12 ng in 0.1 ml, minimized LTB<sub>4</sub> induced TNF synthesis after 1.5 and 3 hours (38 pg/ml and 51 pg/ml) as well as the delayed diapedesis after 4 hours (12 PMN/ml) (all  $p < 0.05$ ). Similarly, pretreatment of other rats by lavage of TNF- $\alpha$  antiserum (rabbit anti-murine), but not normal serum, limited LTB<sub>4</sub> induced diapedesis (13 PMN/ml) ( $p < 0.05$ ). Interestingly, administration of the protein synthesis inhibitor actinomycin D by lavage 10 min after LTB<sub>4</sub> did not prevent TNF generation after 1.5 or 3 hours (490 pg/ml and 440 pg/ml). However, this agent did limit PMN diapedesis after 4 hours (14 PMN/ml) ( $p < 0.05$ ). In contrast, pretreatment of rats by lavage with actinomycin D was without any effect on N-formyl-methionyl-phenylalanine ( $10^{-8}$  M) induced diapedesis. This agent is known to induce PMN migration without need for synthesis of endothelial adhesion proteins. The data indicate that lavage of LTB<sub>4</sub> induces local TNF- $\alpha$  generation that in turn mediates a delayed PMN diapedesis. This event is likely regulated by endothelial synthesis of adhesion proteins.

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## ABSTRACT

In experimental models of acute respiratory failure leukotriene (LT) B<sub>4</sub> is generated in the lungs, followed by a 2 to 3 hour delay before there is substantial neutrophil (PMN) accumulation and increased permeability. This study tests whether lavage of LTB<sub>4</sub> induces tumor necrosis factor (TNF) synthesis by the lungs that in turn mediates PMN diapedesis. Anesthetized rats underwent lavage of 0.1 ml LTB<sub>4</sub> (10<sup>-6</sup>M) into a lung segment. This led to localized TNF synthesis measured in bronchoalveolar lavage fluid (BAL) with peak concentrations of 580 pg/ml after 1.5 hours and 120 pg/ml after 3 hours. These values were higher than following lavage of 0.1 ml saline, 0.7 pg/ml and 4.3 pg/ml respectively (both  $p < 0.05$ ). There was a delay before PMN accumulated in BAL fluid ( $\times 10^4$ ). After 30 min the numbers were 2.2 PMN/ml while at 4 hours there was a rise to 40 PMN/ml and at 5 hours 60 PMN/ml, higher than following saline lavage (all  $p < 0.05$ ). Pretreatment of rats by lavage into airways of actinomycin D, 12 ng in 0.1 ml, minimized LTB<sub>4</sub> induced TNF synthesis after 1.5 and 3 hours (38 pg/ml and 51 pg/ml) as well as the delayed diapedesis after 4 hours (12 PMN/ml) (all  $p < 0.05$ ). Similarly, pretreatment of other rats by lavage of TNF- $\alpha$  antiserum (rabbit anti-murine), but not normal serum, limited LTB<sub>4</sub> induced diapedesis (13 PMN/ml) ( $p < 0.05$ ). Interestingly, administration of the protein synthesis inhibitor actinomycin D by lavage 10 min after LTB<sub>4</sub> did not prevent TNF generation after 1.5 or 3 hours (490 pg/ml and 440 pg/ml). However, this agent did limit PMN diapedesis after 4 hours (14 PMN/ml) ( $p < 0.05$ ). In contrast, pretreatment of rats by lavage with actinomycin D was without any effect on N-formyl-methionyl-phenylalanine (10<sup>-8</sup>M) induced diapedesis. This agent is known to induce PMN migration without need for synthesis of endothelial adhesion proteins. The data indicate that lavage of LTB<sub>4</sub> induces local TNF- $\alpha$  generation that in turn mediates a delayed PMN diapedesis. This event is likely regulated by endothelial synthesis of adhesion proteins.

## INTRODUCTION

Leukotriene (LT) B<sub>4</sub> and neutrophils (PMN) have been found to be important mediators of the adult respiratory distress syndrome (ARDS). Thus, in two experimental models of ARDS, that is pulmonary edema following hindlimb ischemia or acid aspiration (1,2), high concentrations of LTB<sub>4</sub> appear in bronchoalveolar lavage (BAL) fluid and plasma and PMN accumulate in the lungs. Inhibition of lipoxygenase activity or neutrophil depletion, will protect animals from reperfusion injury following ischemia as well as from acid aspiration induced pulmonary edema (3-5). Recently, the process of PMN adhesion to endothelium has been shown to be a key step in decreasing vascular barrier integrity. Thus, inhibition of neutrophil adhesion using a monoclonal antibody (mAb) directed against the PMN membrane adhesion receptor, the CD 18 glycoprotein complex, protected the lung from reperfusion injury (6).

Leukotriene B<sub>4</sub> stimulates neutrophils, leading to CD 18 activation and in high concentrations CD 18 upregulation as well as to increased PMN oxidative metabolism (7,8). These events are associated with adhesion of PMN to the endothelium, followed by protein leakage (9). We have reported that diethylcarbamazine, a lipoxygenase inhibitor prevents the neutropenia noted 5 minutes following reperfusion of ischemic hindlimbs as well as the PMN oxidative activity and the delayed lung leukosequestration (3,8). The fact that there is a delay of several hours before substantial lung leukosequestration occurs is surprising since LTB<sub>4</sub> activation of PMN occurs within 5 minutes and is an event associated with rapid adhesion to an *in vitro* endothelial monolayer as well as to the skin microvasculature *in vivo* (7,9). These rapid changes in neutrophil function therefore do not explain the delayed lung inflammatory response associated with LTB<sub>4</sub>. In other reports, aspiration of LTB<sub>4</sub> into sheep lungs promoted PMN diapedesis over a 16 hour time period (10). Similar observations of delayed lung leukosequestration have been noted not only in the setting of hindlimb ischemia but also following acid aspiration. In both settings, the

early rise in plasma LTB<sub>4</sub> preceded by 2 to 3 hours lung neutrophil accumulations. This time interval strongly suggests involvement of another intermediary whose synthesis was stimulated by LTB<sub>4</sub>.

The reported kinetics of PMN adhesion and diapedesis following LTB<sub>4</sub> lung lavage in the sheep (10), mimic the time scale of endothelial activation with cytokines. Thus, an endothelial cell monolayer treated with tumor necrosis factor (TNF) or interleukin-1 (IL-1) is stimulated in a process requiring protein synthesis and therefore, occurring over 2 to 3 hours, to express adhesion receptors such as intercellular adhesion molecule-1 (ICAM-1) or endothelial leukocyte adhesion molecule-1 (ELAM-1) (11,12). Since LTB<sub>4</sub> by itself cannot activate endothelial cells to express adhesion receptors we hypothesize that in vivo this eicosanoid can act indirectly by stimulating cytokine synthesis. Indeed, LTB<sub>4</sub> has been associated with TNF synthesis. Thus, pulmonary macrophages treated with LTB<sub>4</sub> synthesize and release TNF in a dose dependent fashion (13). These cytokines are not stored and agents or events that lead to their production require preliminary protein synthesis by the manufacturing cell (11). Further, that TNF plays an important role in determining PMN-endothelial interactions has been reported in endotoxemia (14) and following administration of recombinant TNF to rats and mice (15,16). In addition, this cytokine has been found to determine neutrophil adhesion in the setting of HCl aspiration (17).

We tested the thesis that LTB<sub>4</sub> lavaged in the lungs leads to delayed PMN diapedesis by stimulating synthesis of a cytokine intermediary, TNF. The results show that lavage of LTB<sub>4</sub> leads to TNF- $\alpha$  synthesis that in turn mediates later neutrophil diapedesis. The data indicate further that this event is regulated by endothelial synthesis of adhesion proteins.

## METHODS

### Animal Preparation

Adult male Sprague-Dawley rats (Charles River Lab, Wilmington, MA) (n=114) weighing approximately 500 g were anesthetized with intraperitoneal ketamine (35 mg/kg). A jugular venous catheter was inserted for fluid infusion (1.5 ml/h) and hourly intravenous anesthetic dosing (ketamine, 8 mg/kg; xylazine 1 mg/kg). A tracheostomy was performed with a 15-gauge tube. Through this tube, a fine-bore polyester cannula with an external diameter of 0.61 mm and an internal diameter of 0.28 mm was introduced into the anterior segment of the left lung which represented approximately one-third the weight of the left lung. Cannula positioning was uncomplicated and based on the normal trajectory of a soft tube.

### Preparation of Solutions

*Leukotriene B<sub>4</sub>*: a stock solution of 0.1 mg/ml ( $3.0 \times 10^{-4}$ M, Sigma, St. Louis, MO) was made up using methanol. Saline was used for dilution to a final concentration of  $10^{-6}$ M.

*N-formyl-methionyl-leucyl-phenylalanine (FMLP)*: (Sigma) a stock solution of 5mg/ml ( $10^{-2}$ M) was dissolve in saline to a final concentration of  $10^{-8}$ M. This agent was used as a control. It induces PMN diapedesis via upregulation of neutrophil CD 18 and not by activation of endothelium to synthesize adhesion molecules (22).

*Actinomycin D*: a stock solution of 1 mg/ml was dissolved in phosphate buffered saline to a final concentration of 0.12 µg/ml.

*Rabbit anti-murine TNF-α anti-serum* (IP:400, Genzyme Corporation, Boston, MA) was derived from New-Zealand rabbits immunized with recombinant murine TNF-α. The serum was sterilized using 0.22 micron filter. It has a neutralizing activity of approximately  $10^6$  TNF-α units per ml and has no cross-reactivity with TNF-β, IL-1 or gamma interferon. The stock solu-

tion of 1 ml was diluted 1:25 with saline and 0.1 ml was lavaged into airways.

*Rabbit Serum:* Sterile normal rabbit preimmune serum (IP:001 Genzyme) was diluted and given in the same volume as IP:400. Endotoxin content of IP:001 or IP:400 was less than 1 pg/ml in the volume of serum administered, as assayed with the Limulus ameocyte lysate (Associates of Cape Cod, Falmouth, MA).

#### TNF Assay

TNF activity was measured using a bioassay whose end point is cytotoxicity (18). The WEHI 164 clone 13 line was used as target cells (19). Serial, log 2 dilutions of BAL fluid samples were made (Roswell Park Memorial Institute solution mixed with 10% fetal calf serum) and were then added to target cells ( $4 \times 10^4$  cells/well) in a final volume of 0.1 ml in the presence of actinomycin D (1  $\mu$ g/ml). After culture at 37°C for 18 hours, the supernatants were discarded and the plates washed by immersion in warm phosphate buffered saline (PBS). The remaining viable adherent cells were stained with crystal violet (0.2% in 2% methanol) for 5 minutes. After three rinses with PBS, the absorbance at 610 nm was read using an automated plate spectrophotometer (VMax, Molecular Devices, Palo Alto, CA). Each plate included serial dilutions of purified recombinant TNF- $\alpha$  (Genzyme, Boston). The concentration of TNF- $\alpha$  activity in unknown samples was derived by regression analysis using values falling in the range giving 20-80% of maximal lysis as established by the TNF- $\alpha$  standards.

#### Experimental Protocol

Evans Blue dye 0.2 mg was added to all the solutions for later confirmation of the lung segment lavaged. In each case, two solutions, an initial and a secondary were administered into the left inferior anterior lung segment at 10 minute intervals via the fine bore intrabroncheal can-

nulae in a volume of 0.1 ml each. There were eight experimental groups. Each group was defined according to the first and second agent lavaged: saline-saline (n=26), saline-LTB<sub>4</sub> (n=38), actinomycin D-LTB<sub>4</sub> (n=14), IP:400-LTB<sub>4</sub> (n=6), IP:001-LTB<sub>4</sub> (n=6), LTB<sub>4</sub>-actinomycin D (n=14). Finally, in order to test whether LTB<sub>4</sub> induced lung diapedesis is unique, two additional groups were used: saline-FMLP (n=5), and actinomycin D-FMLP (n=5).

The intrabronchial cannulae was removed after the second treatment. Every thirty minutes over a period of 5 hours, 4 to 6 animals were euthanased with overdose of ketamine. A thoracotomy was performed and the right lung bronchus clamped. Bronchoalveolar lavage was performed with 3 ml of saline, using the tracheostomy tube. It was repeated three times with gentle lung massage. The combined lavage return of about 8 ml was centrifuged at 7,000 x g for 5 minutes. The supernatant was frozen in 1 ml aliquots at -20° and subsequently used to assay TNF. The pellet was suspended in 1 ml saline and PMN counted in a blind fashion after Diff-Quik staining to identify macrophages (AHS del Caribe, Inc., Aguda, Puerto Rico). The results are expressed as PMN/ml (mean ± standard error x 10<sup>4</sup>). Four to six rats from each time point and experimental group were used for TNF and/or PMN count in BAL fluid.

Results are presented as mean ± SEM in text, Figures and Table. The difference between means was tested by an analysis of variance, and if significance was found, by a non-paired Student's t-test. Significance was accepted if p < 0.05.

Animals in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, Department of Health, Education and Welfare, Publication No. 78-23 (National Institute of Health), revised, 1978.

## RESULTS

When the initial treatment was saline,  $\text{LTB}_4$  subsequently lavaged into airways led to TNF synthesis as measured in BAL fluid,  $580 \pm 230$  pg/ml after 1.5 hours and  $120 \pm 60$  pg/ml after 3 hours. Both values were higher than the saline-saline group  $0.7 \pm 0.2$  pg/ml and  $4.3 \pm 3$  pg/ml ( $p < 0.05$ , Fig. 1). After 5 hours TNF concentration in BAL fluid had returned to values similar to the saline-saline group. Saline- $\text{LTB}_4$  lavage led to PMN accumulations in BAL fluid starting after 30 minutes  $2.2 \pm 0.5 \times 10^4$  PMN/ml which increased over the 5 hours of monitoring to  $60 \pm 6 \times 10^4$  PMN/ml ( $p < 0.05$ , Fig. 2). When the protein synthesis inhibitor actinomycin D was given by lavage before  $\text{LTB}_4$ , TNF generation in BAL fluid was markedly reduced after 1.5 and 3 hours (Table 1). In addition, neutrophil accumulations in BAL fluid were reduced to  $12 \pm 2 \times 10^4$  PMN/ml after 4 hours, a value lower than lavage with saline- $\text{LTB}_4$   $40 \pm 5 \times 10^4$  PMN/ml (both  $p < 0.05$ , Fig. 3).

Lavage with TNF- $\alpha$  anti-serum prior to  $\text{LTB}_4$  also reduced diapedesis to  $13 \pm 2 \times 10^4$  PMN/ml ( $p < 0.05$ ) while lavage with normal serum was without effect  $43 \pm 3 \times 10^4$  PMN/ml. When  $\text{LTB}_4$  was lavaged first followed in 10 minutes by actinomycin D, BAL fluid TNF levels were still high  $490 \pm 100$  pg/ml after 1.5 hours and  $440 \pm 160$  pg/ml after 3 hours (Table 1). However, at 4 hours few neutrophils accumulated in BAL fluid  $14 \pm 2 \times 10^4$  PMN/ml ( $p < 0.05$ ). Finally, saline-FMLP led to BAL fluid accumulations of  $19 \pm 2 \times 10^4$  PMN/ml after 4 hours, an event that was not affected by pretreatment with actinomycin D before FMLP  $24 \pm 5 \times 10^4$  PMN/ml.

## DISCUSSION

The data indicate that segmental lung lavage of  $\text{LTB}_4$  induces a delayed PMN diapedesis mediated by TNF- $\alpha$ . Thus, elevated levels of TNF appear in BAL fluid prior to neutrophil accu-

mulations (Fig 1 and 2). Inhibition of TNF synthesis with actinomycin D or TNF activity with TNF- $\alpha$  antiserum prevents the delayed neutrophil migration into the lungs (Fig. 3).

LTB<sub>4</sub> leads to both an early and late flux of neutrophils into alveolar spaces. Thus, 30 and 60 minutes following LTB<sub>4</sub> lavage there were more PMN in BAL fluid relative to control values (Fig. 2). This rapid event is likely mediated by LTB<sub>4</sub> activation of marginating lung neutrophils. Neutrophil activation by this eicosanoid occurs over minutes and does not require protein or cytokine synthesis (20,22). Indeed, inhibition of protein synthesis with actinomycin D or TNF- $\alpha$  activity with antiserum did not eliminate all PMN accumulations. Thus, in these animals, after 4 hours the number of cells recovered in BAL fluid, 12 to 13 x 10<sup>4</sup> PMN/ml was equivalent to the number recovered in saline-LTB<sub>4</sub> treated animals after 2 hours (Fig. 3). This early migration of neutrophils is likely independent of cytokine and protein synthesis. A similarly, rapid influx, within 5 minutes of neutrophils into the alveolar space has been reported following lavage of LTB<sub>4</sub> in the guinea pig (21). Further, injection of LTB<sub>4</sub> into rabbit skin also led to early diapedesis, an event shown to be regulated by neutrophil CD 18 expression and not endothelial synthesis of adhesion proteins (9,22). However, in contrast to the skin inflammatory response that subsides 1 to 3 hours after LTB<sub>4</sub> injection, lung neutrophil accumulations are progressive over the 5 hour period of monitoring (Fig. 2). The reason(s) for these site specific differences is unknown but is similar to reported differences between diapedesis in the lungs and peritoneum (23)

The later neutrophil accumulations in BAL fluid in the current study are in accord with other reports demonstrating that aspiration of LTB<sub>4</sub> into sheep airways stimulates neutrophil diapedesis over a 16 hour period of monitoring (10). The lag time of about 2 hours is likely the period required first for TNF synthesis and then for endothelial activation and synthesis of adhesion proteins. Thus, LTB<sub>4</sub> cannot itself activate endothelial cells to express adhesion mole-

cules (24). However, it has been reported that lipoxigenase activity is associated with cytokine synthesis in vitro (25,26). In the present study a marked rise of TNF appeared 1.5 hours following lavage of LTB<sub>4</sub> into lung airways (Fig. 1). This finding is consistent with in vitro experiments showing that lung monocytes in culture synthesize TNF when treated with LTB<sub>4</sub> (13).

The data indicate that TNF- $\alpha$  generated by LTB<sub>4</sub> stimulation of lung parenchyma activates endothelium of the microvasculature, leading to delayed diapedesis. This conclusion is based on the ability of actinomycin D to prevent the synthesis of TNF and perhaps other cytokines along with reduction in PMN diapedesis. The equal effectiveness of TNF- $\alpha$  antiserum and actinomycin D in preventing PMN alveolar flux confirms TNF- $\alpha$  as the prime intermediary and makes other cytokine involvement in this setting less likely.

Interestingly, administration of a protein synthesis inhibitor 10 minutes before but not after LTB<sub>4</sub> was capable of preventing TNF synthesis (Table 1). However, despite the inability to inhibit TNF synthesis, actinomycin D prevented PMN migration. This observation is in accord with in vitro studies showing the importance in the process of PMN endothelial interaction of other types of protein synthesis. Thus, actinomycin D will prevent TNF or IL-1 induction of endothelial adhesion molecules such as ICAM-1 or ELAM-1 (11,27). That actinomycin D did not prevent FMLP induced alveolar diapedesis indicates first, that endothelial activation is not mandatory for neutrophil diapedesis in response to certain chemoattractants. Thus, FMLP induces diapedesis by stimulating neutrophil adhesion receptors which in turn interact with basally expressed endothelial adhesion molecules (20,22). Secondly, actinomycin D does not exert its effect by direct inhibition of PMN migration. Other reports confirm the absence of an effect of actinomycin D on PMN movement (28). The current study cannot exclude the possibility that FMLP in different concentrations may activate lung cells to synthesize cytokine.

The ability of LTB<sub>4</sub>-actinomycin D treatment to limit diapedesis but not TNF synthesis argues against the possibility that TNF stimulates further chemoattractant release such as complement fragments, platelet activating factor, peptidoleukotrienes or thromboxane A<sub>2</sub>, agents that can induce PMN-endothelial interactions independent of protein synthesis (22). Further, reduced diapedesis with high levels of TNF also make unlikely the possibility that the TNF effect is via upregulation of PMN CD 18, an action which is also independent of protein synthesis. The finding that PMN diapedesis into the alveolar space progressively increased following the peak of TNF synthesis is not surprising. It has been found that once endothelial cells are activated with TNF, their adhesion molecules will remain expressed over 8 to 24 hours (11,12).

These results may explain similar phenomena observed in other experimental models of ARDS. For example, there is a lag period between the rise in plasma LTB<sub>4</sub> and neutrophil accumulations in lungs following remote ischemia or after localized acid aspiration (2,3). The involvement of TNF in HCl aspiration has been confirmed by the observations that TNF- $\alpha$  antiserum will limit the delayed neutrophil accumulations in the lungs, as will inhibition of protein synthesis with cycloheximide. This unique LTB<sub>4</sub>-TNF pathway in the lungs might be responsible for the sustained leukocyte infiltration into lungs of patients with ARDS. These cells which release elastase and free radicals may thereby mediate the respiratory failure (29).

In summary, these data indicate that lavage of LTB<sub>4</sub> into airways induces local TNF synthesis that in turn mediates delayed alveolar neutrophil diapedesis, an event regulated by endothelial synthesis of adhesion proteins.

Figure 1. Lavage of LTB<sub>4</sub> into lung airways induced local TNF synthesis in BAL fluid. The symbol \* indicates  $p < 0.05$  relative to the saline-saline group.

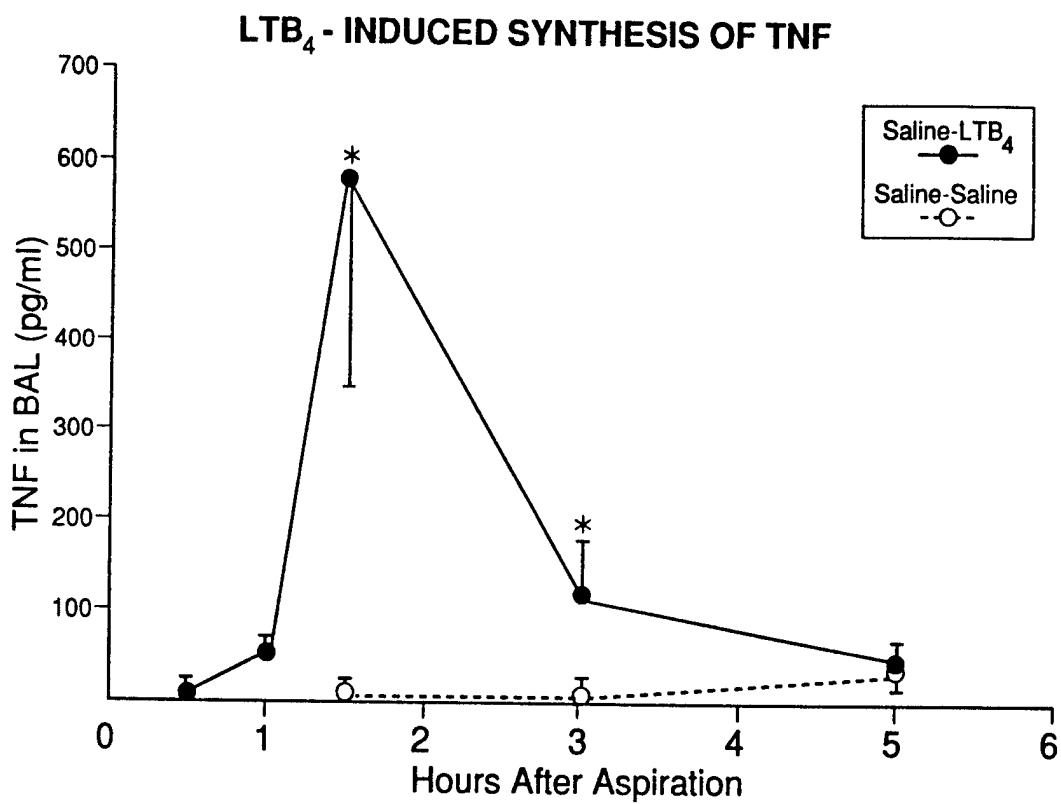


Figure 2. Neutrophil accumulations in BAL fluid in response to  $\text{LTB}_4$  lavage rose progressively over the 5 hours of monitoring. This event was significantly ( $p < 0.05$ ) higher at each time point relative to saline-saline lavage.

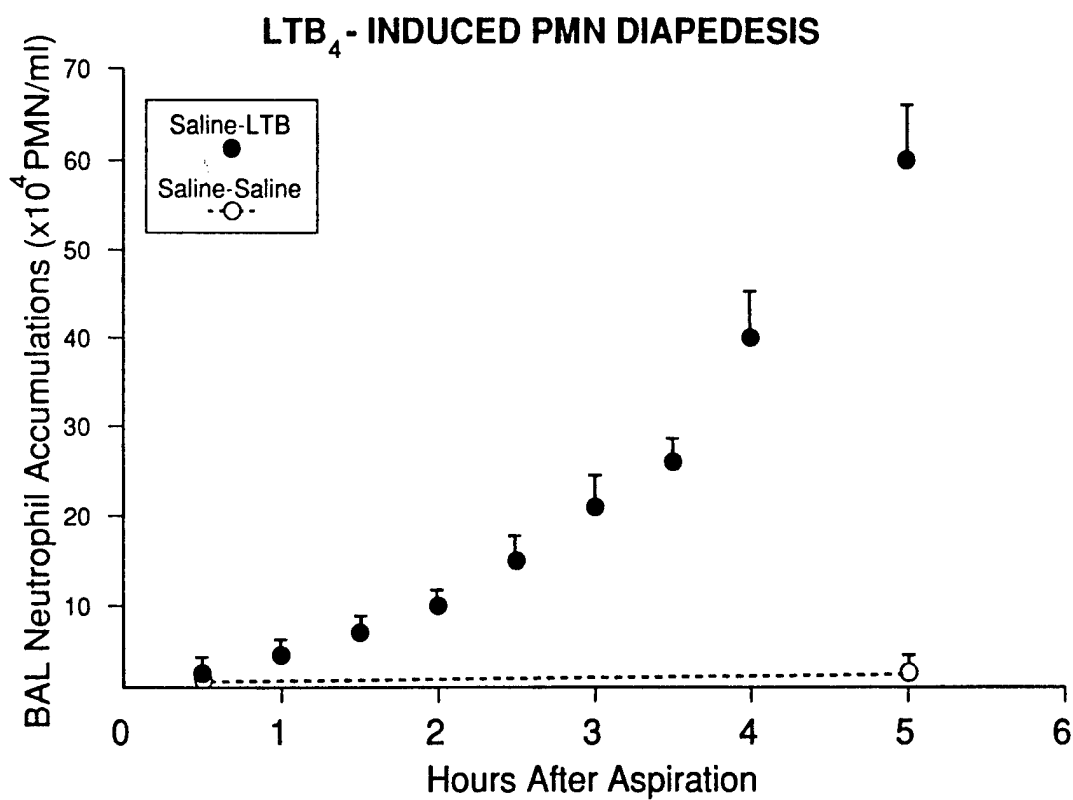
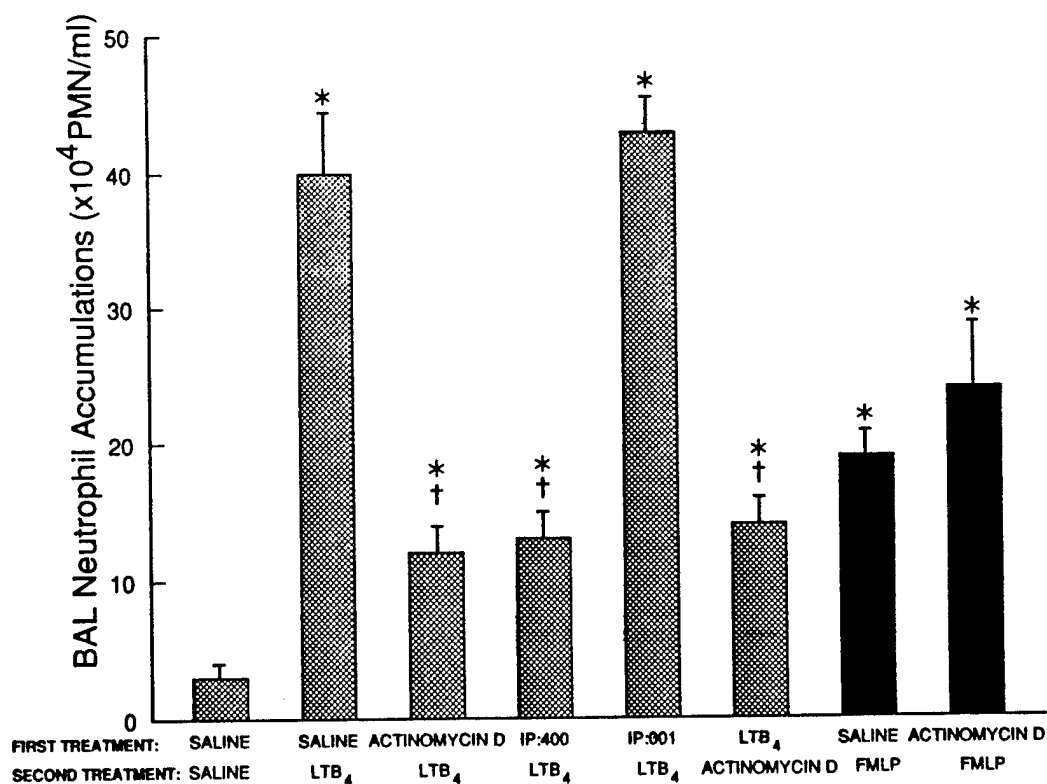


Figure 3. Lavage of saline-LTB<sub>4</sub> into airways led to delayed PMN accumulations in BAL fluid after 4 hours. Diapedesis was reduced by lavage of actinomycin D before (when TNF synthesis was inhibited) or after LTB<sub>4</sub> lavage. The latter sequence is consistent with inhibition of endothelial synthesis of adhesion proteins. TNF- $\alpha$  antiserum (IP:400) but not control serum (IP:001) minimized LTB<sub>4</sub> induced diapedesis. In contrast, PMN diapedesis induced by FMLP lavage was not affected by actinomycin D. The symbols \* and † indicate  $p < 0.05$  relative to saline-saline and saline-LTB<sub>4</sub> respectively.



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TABLE 1

EFFECT OF LTB<sub>4</sub> AND ACTINOMYCIN D ON LUNG SYNTHESIS OF TNF

Hours After Aspiration Group	TNF Concentration (pg/ml)	
	1.5	3
Saline-Saline	0.7 ± 0.2	4.3 ± 3
Saline-LTB <sub>4</sub>	580 ± 230*	120 ± 60*
Actinomycin D-LTB <sub>4</sub>	38 ± 18*†	51 ± 14*
LTB <sub>4</sub> -Actinomycin D	490 ± 100*	440 ± 160*†

\* and † indicate  $p < 0.05$  relative to saline-saline and saline-LTB<sub>4</sub> respectively.